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13. ABSTRACT (Maximum 200 Words) Breast cancer is the leading cause of female cancer deaths in this country and the incidence of new cases continues to rise. Vitamin A status is a possible factor contributing to the development of many human cancers, including those of the breast. A major question regarding the role of vitamin A and breast cancer remains unanswered. How, on a molecular level, do retinoids induce growth arrest of hormone-dependent breast cancer cells? I proposed to test the hypothesis that "biologically active derivatives of vitamin A (retinoids) inhibit mammary carcinoma cell proliferation by disrupting one or more growth factor activated serine/threonine protein kinase signaling cascades. Targets of these signaling cascades include genes that encode proteins required for progression through the cell cycle" . Specifically, I proposed to identify and isolate genes whose expression is regulated by retinoic acid in hormone-dependent, but not hormone-independent cells, and determine if these genes encode proteins involved in cell cycle progression. As a result of these studies we demonstrated that: <ul style="list-style-type: none"> • PKCα and PTP-1C are up-regulated in retinoid treated cells • PKCα mediates the anti-proliferative action of retinoids in T47D cells • PKCα cooperates with retinoids to limit the proliferation of the normally retinoid resistant cell line, MDA-MB-231 In the long term, these results might open the door for developing combination therapy approaches that jointly target retinoid and PKC signaling.				
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INTRODUCTION

Breast cancer is the leading cause of female cancer deaths in this country and the incidence of new cases continues to rise. The reasons for this increase are unclear. Genetic cases account for only about 10% of breast cancers. A better understanding of other causative factors is required for developing alternative or additional therapies, and is required for the development and implementation of preventive interventions. Vitamin A status is a possible factor contributing to the development of many human cancers, including those of the breast. Epidemiological studies, experiments with animal models and clinical trials have implicated vitamin A status as an important factor in the development of a variety of human cancers (1-6). These studies establish a link between dietary retinoid intake and cancer risk, and further show that retinoids can prevent progression of some, but not all, human cancers. Despite these advances, a major question regarding the role of vitamin A and breast cancer remains unanswered. How, on a molecular level, do retinoids induce growth arrest of hormone-dependent breast cancer cells? I had originally proposed to test the hypothesis that **“biologically active derivatives of vitamin A (retinoids) inhibit mammary carcinoma cell proliferation by disrupting one or more growth factor activated serine/threonine protein kinase signaling cascades. Targets of these signaling cascades include genes that encode proteins required for progression through the cell cycle”**. The experimental approach was designed to identify the retinoid-regulated genes that negatively regulate cell cycle progression. Specifically, I proposed to identify and isolate genes whose expression is regulated by retinoic acid in hormone-dependent, but not hormone-independent cells, and determine if these genes encode proteins involved in cell cycle progression.

BODY

The initial proposal involved using a modified “enhancer trap” strategy to identify and molecularly clone RA-induced genes whose products were involved in negative regulation of cell cycle progression (7,8). This approach had to be abandoned in Year 2 as a result of intractable technical difficulties. These difficulties have been discussed extensively in the Year 1 and Year 2 Progress reports.

Using alternative approaches we have identified a critical retinoic acid induced gene, that encoding the alpha isoform of protein kinase C (PKC α). We also have identified a gene whose expression is required for RAR α function in the T47D breast cancer cell line, and have evidence for a possible target of PKC α . These genes are the cellular retinoic acid binding protein type 2 (CRABP 2) and the protein phosphotyrosyl phosphatase 1C (PTP-1C).

Progress on the original “Statement of Work”:

Technical Objective 1: Insertional mutagenesis and molecular tagging of RAR α -induced genes using an “enhancer trap” retroviral vector. The original goal of Technical objective 1 was to develop and employ an “enhancer trap” based strategy for cloning RA-induced genes in the T47D breast cancer derived cell line. As described in detail in previous progress reports, we found that the T47D cell line is refractory to infection with recombinant retroviruses. Attempts to restore expression of retrovirally encoded genes failed and as a result, we were forced to abandon our originally proposed strategy for isolating RA-induced genes.

Task 1: Construct pLLGFPSV and pRARE-tk-GFP & Task 2: Establish conditions for FACS of GFP expressing cells. These tasks were completed in years 1 and 2.

Task 3: Generate NIH3T3/GP+E-Amph12 packaging cells producing LLGFPSV. Although not completed as originally proposed we were able to package recombinant retroviruses following transient infections of a HEK293T-based packaging cell line (9).

Task 4: Isolate T47D cells expressing GFP in an RAR-dependent manner. We failed in this task as detailed in previous years, and consequently abandoned **Task 5: Analyze RAR-regulated genes using pLLTX and pRSV-Cre.**

Technical Objective 2: Clone RAR α -induced growth suppressing cDNAs.

We identified 2 proteins whose expression was induced following RA treatment of T47D cells. The first is the alpha isoform of protein kinase C (**Figure 1** and ref. 10). The second is an SH2 domain containing, non-receptor tyrosine phosphatase known as PTP-1C (**Figure 2** and ref. 11). We have constitutively expressed PKC α in T47D cells. PKC α expressing cells initially grew slowly, did not respond to further treatment with retinoic acid and eventually stopped growing altogether. We are currently establishing T47D cells in which PKC α will be under the control of a modified tetracycline responsive transactivator ("Tet-Off"). Further experiments demonstrated that inhibiting PKC α activity (with Gö6976) or expression (with antisense oligonucleotides), either in RA treated T47D cells, or in T47D-PKC α cells, significantly increased proliferation. In the course of studies on how RA and PKC α inhibit proliferation we noted elevated levels of a ~60 kD phosphotyrosine containing protein in RA-treated cells. A screen of possible candidates led to the tentative identification of this protein as PTP-1C. Levels of PTP-1C are elevated in RA-treated T47D cells and in T47D cells expressing PKC α . Based on these results we have focused on the role of PKC α as a critical RA-induced target gene. In addition we have initiated studies aimed at determining if PTP-1C is also critical for the RA-induced phenotype.

Task 6: Construct T47D cDNA library. This task was never initiated.

Task 7: Clone RAR α -regulated cDNAs. We have identified 2 genes whose expression is up regulated following RA treatment of T47D cells. A similar response was not seen in retinoid resistant MDA-MB-231 cells. These 2 are protein kinase C α and the SH2 domain containing tyrosine phosphatase PTP-1C.

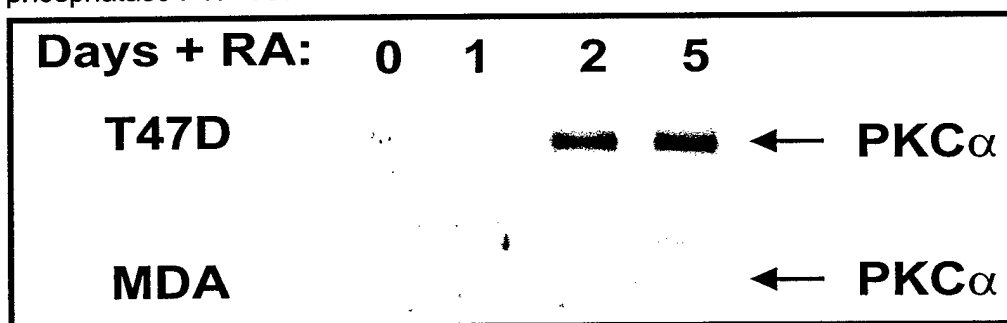


Figure 1. Retinoic acid induces protein kinase C α . T47D (sensitive to growth inhibition by RA) or MDA-MB-231 (insensitive to RA) cells were grown in the presence of 10^{-6} M all trans retinoic acid (RA) for the indicated times. Whole cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with antibodies specifically recognizing PKC α . RA induced PKC α expression in T47D but not MDA-MB-231 cells.

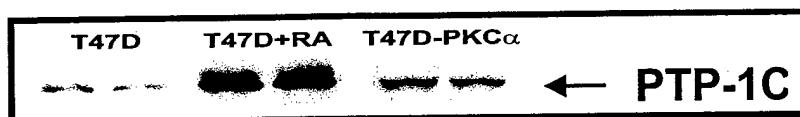


Figure 2. RA and PKC α expression induce PTP-1C levels. T47D cells were treated with solvent (T47D), 10^{-6} M RA (+RA, 48 hrs) or stably transfected with a vector encoding PKC α (T47D-PKC α). Whole cell lysates (30 μ g total protein in each lane) were resolved by SDS-PAGE, transferred to nitrocellulose and probed with anti-PTP-1C antibodies. Duplicate treatments are shown for each condition. Both RA treatment and PKC α expression increased steady-state levels of PTP-1C.

Technical Objective 3: Characterization of function of RAR α -regulated genes.

Task 8: Determine if antisense cDNAs confer retinoid resistance on T47D cells. This task was completed in Year 3. Initially I had proposed to stably express antisense RNAs in T47D cells. However, because of the exceedingly low efficiency of stable transfection in these cells, we choose to use antisense oligonucleotides. Proliferation assays were performed on T47D cells treated with 10^{-9} and 10^{-6} M RA in the presence of antisense oligonucleotides and mismatch oligonucleotides directed at PKC α , PTP-1C and CRABP 2. Treatment with the antisense oligonucleotides completely (PKC α and CRABP 2) or partially (PTP-1c) restored proliferation in the presence of RA. These results confirm that PKC α is an essential mediator of the anti-proliferative activity of RA in T47D cells, and point to a role for PTP-1C as well. In addition, these results demonstrate that CRABP 2 is an essential component of the RA-signaling pathway in these cells. The possible importance of this finding is that CRABP 2 expression is lost in many, RA-resistant breast cancer cell lines (12).

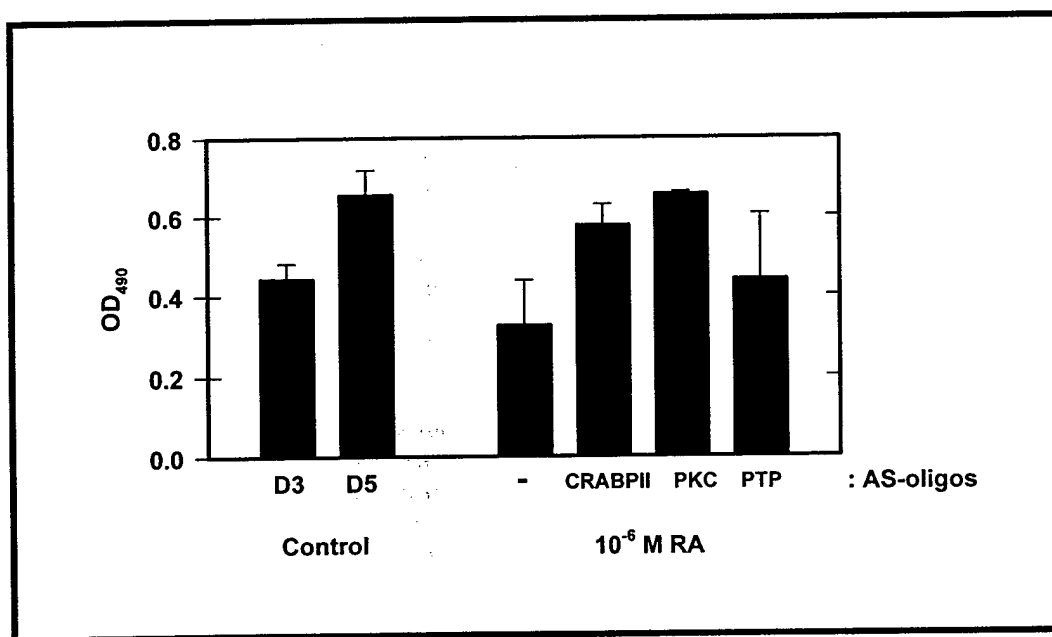


Figure 3. Antisense oligonucleotides against CRABP 2, PKC α and PTP-1C counteract the effects of RA. T47D cells were plated in 96 well dishes at 5000 cells/well and treated with 10^{-6} M retinoic acid (RA) or antisense oligonucleotides (AS-oligos, 10 μ M) for 5 days. Viable cell numbers were determined by measuring the extent of MTS reduction spectrophotometrically (OD₄₉₀). Results for untreated cells are shown after 3 (D3) and 5 (D5) days. The effects of CRABP 2 and PKC α AS-oligos were significant at $p < 0.05$; the effect of AS-PTP-1C was not.

Oligo sequences:

Antisense PTP-1C: 5'-GGACT TACCC ACGGG ACAGC ATCTC-3'
Antisense PKC α : 5'-CGTTG CCCGG GAAAA CGTCA GCCAT GGTC-3'
Antisense CRABP 2: 5'-CAGTT GCCAG AGAAGT TGGGC ATG-3'

Task 9: Establish the effect of constitutive expression of cDNAs in T47D and MDA-MB-231 cells. This task has been completed for PKC α . The results are described in three manuscripts, one published (10) and two submitted for publication (currently under revision). Both T47D and MDA-MB-231 derived cell lines have been isolated that constitutively express a PKC α cDNA. Expression has been confirmed by immunoblot analysis and by enzymatic assays (ref. 10). As noted above, constitutive expression of PKC α in T47D cells slows proliferation (to an extent equivalent to treatment with 10^{-8} M RA)(10). Detailed studies on RA-induced growth arrest of T47D cells demonstrated that: (1) RA increased population doubling times from 36 to >100 hr, without decreasing cell viability; (2) RA treatment significantly reduced EGF activation of the EGF receptor without altering EGFR expression levels (**Figure 4**); (3) RA treatment attenuated EGF signaling to the nucleus via MAPK cascades (**Figure 5**). The effect of RA on EGFR activation and MAPK activation was totally reversed by prior treatment of cells with an inhibitor of PKC α . In addition to reduced proliferation (resulting from increased population doubling times and not cell death) T47D-PKC α cells were largely refractory to stimulation with EGF at the level of EGFR activation and MAPK activation.

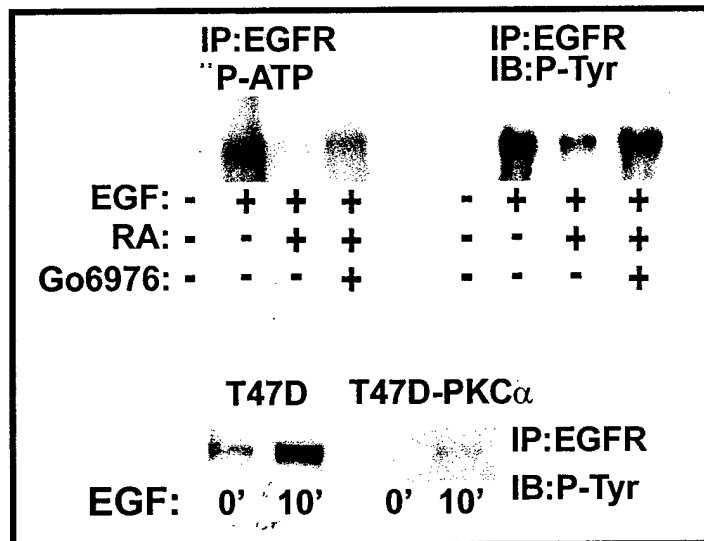


Figure 4. Retinoic acid treatment and PKC α expression attenuate activation of the EGF receptor. Top: T47D cells were treated overnight with 10^{-6} M RA and then stimulated for 10 min with 40 ng/ml EGF. Where indicated the conventional PKC inhibitor, Gö6976, was added 30 min prior to EGF stimulation. EGFR was immunoprecipitated and either incubated with 32 P- γ -ATP or immunoblotted with antiphosphotyrosine antibodies (P-Tyr). RA pretreatment partially inhibited EGFR activity and the RA block was reversed when PKC α was inhibited. Bottom: T47D cells and PKC α expressing T47D cells were stimulated with 40 ng/ml EGF for 0 or 10 min and the EGFR was immunoprecipitated and subjected to antiphosphotyrosine blotting. PKC α expression inhibited both basal (0 min) and stimulated tyrosine phosphorylation of the EGFR.

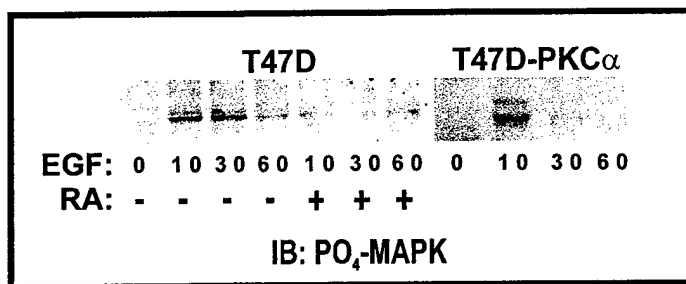


Figure 5. Both RA treatment and PKC α expression inhibit activation of MAPK by EGF. T47D or T47D-PKC α expressing cells were stimulated with 40 ng/ml EGF for 0 – 60 min. Where indicated T47D cells were pretreated overnight with 10⁻⁶ M RA. Whole cell lysates (30 μ g protein/lane) were resolved by SDS-PAGE, transferred to nitrocellulose and probed with an antibody that specifically recognizes the active forms of MAPK 1 and 2. RA treatment limited the magnitude and duration of MAPK activation, whereas PKC α expression predominantly limited the duration of MAPK activation.

Constitutive expression of PKC α in MDA-MB-231 cells (**Figure 6**) did not alter proliferation (**Figure 7**) or fetal bovine signaling to the nucleus (**Figure 8**). Therefore the lack of retinoid responsiveness of this cell line is not simply the result of the failure of RA to induce PKC α expression. Surprisingly (and of great interest), when PKC α expressing MDA-MB-231 cells were treated with RA, there was a significant decrease in proliferation (**Figure 7**), colony forming efficiency (not shown; ref 13), and serum induction of c-fos expression and MAPK activation (**Figures 8 & 9**). The role of PKC α in these changes in the response to RA were confirmed by expressing PKC α under a promoter in which expression was reversibly induced with IPTG (13). In these cells, RA responsiveness appeared ~24 hrs after adding IPTG to the media and persisted until IPTG was removed. After an additional 24 hrs, both PKC α (detected by immunoblotting) and RA responses were absent. The basis for the PKC α -dependent response to RA is not known.

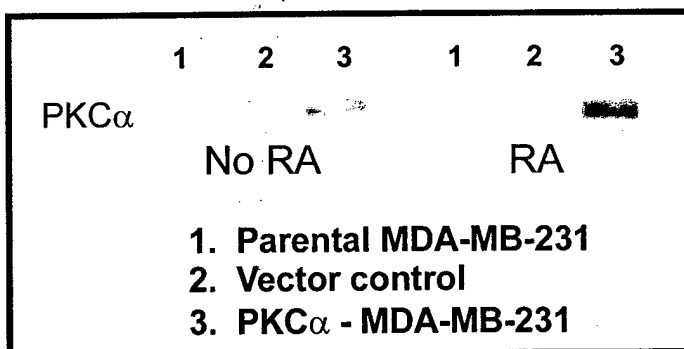


Figure 6. MDA-MB-231 cells were infected with recombinant retroviruses encoding G418 resistance (vector control) or PKC α and G418 resistance (PKC α -MDA-MB-231). Stable G418 resistant cell lines were isolated and protein kinase C expression was measured by immunoblotting. PKC α was only detected in cells infected with the PKC α encoding retrovirus (cell line 3). In addition, constitutively expressing PKC α did not alter the expression profile of other PKC isoforms.

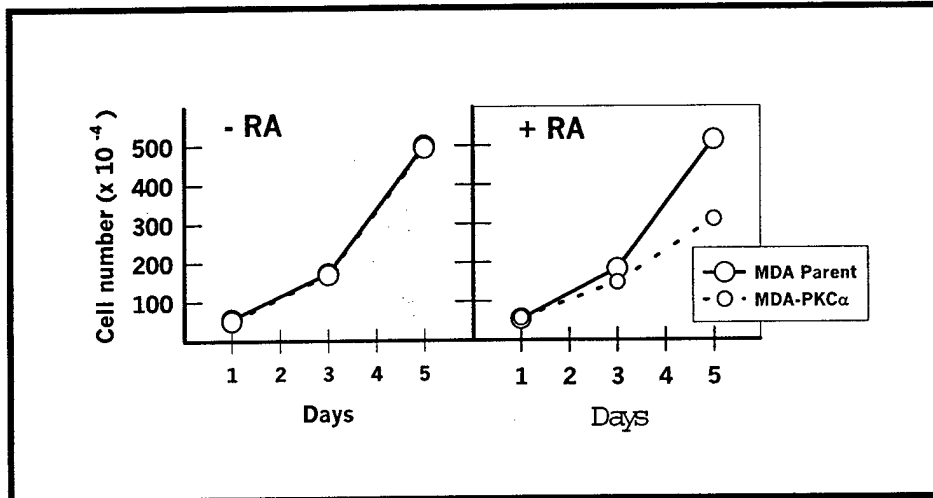


Figure 7. PKC α expression confers retinoid sensitivity on MDA-MB-231 cells. The growth rate of MDA-MB-231 cells (filled circle) and PKC α expressing MDA-MB-231 cells (open circles) was compared in the absence (-RA) or presence of 10^{-6} M retinoic acid (+RA). Neither PKC α expression nor RA treatment affected MDA-MB-231 proliferation. However, RA treated PKC α expressing cells grew at a reduced rate.

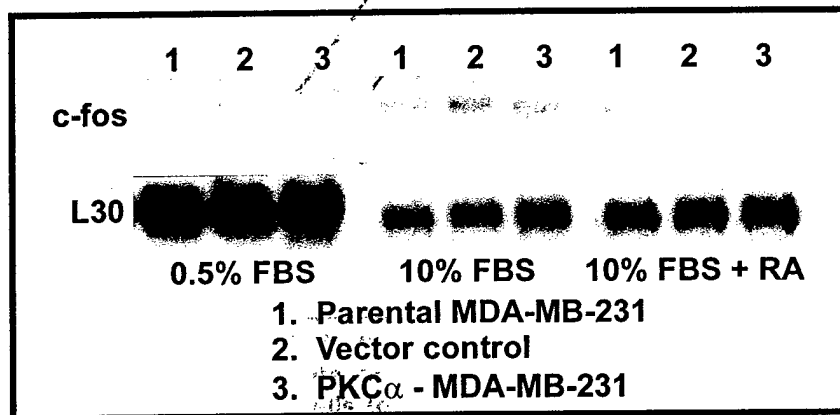


Figure 8. The combined anti-proliferative effect of PKC α expression and RA treatment is associated with reduced serum induced c-fos expression. This was demonstrated by northern blot analysis of serum starved (0.5% FBS) or serum stimulated (10% FBS, stimulation was for 30 min, $\pm 10^{-6}$ M RA) parental MDA-MB-231 cells (cell line 1), vector control cells (line 2) or PKC α expressing MDA-MB-231 cells (line 3). Only in the PKC α expressing cells did RA inhibit the serum induction of c-fos. In contrast c-jun expression was elevated in PKC α expressing cells under all conditions, and RA inhibited induction of junB in all 3 cell lines.

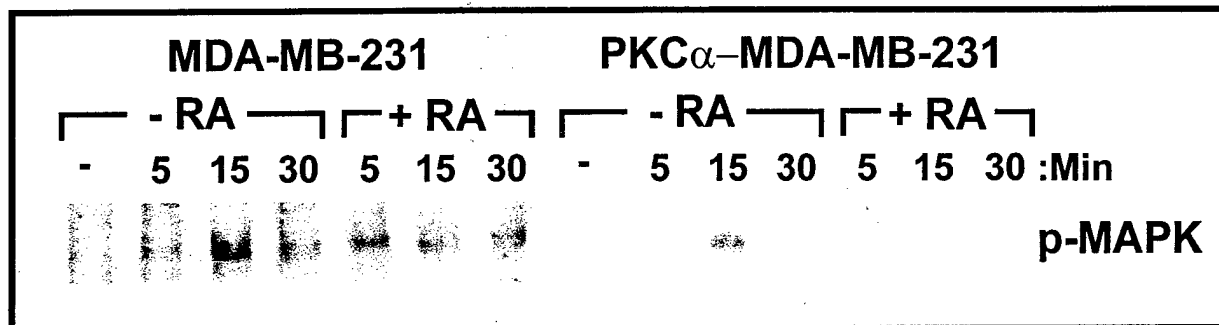


Figure 9. PKC α expression also markedly attenuated serum induced MAPK activity. Serum activation of MAPK kinase was determined by immunoblotting. Whole cell extracts of MDA-MB-231 cells (A) or PKC α -MDA-MB-231 cells (B) after 0 – 30 min stimulation with 10% fetal bovine serum were probed with antibodies specific for the activated form of MAPK (Phospho-MAPK). Filters were stripped and re-blotted with antibodies that recognize both active and inactive MAPK (Total MAPK). In parental MDA-MB-231 cells RA had no effect on the magnitude or the duration of serum induced MAPK activity. In contrast, in PKC α -MDA-MB-231 cells the response to serum was reduced and when cells were co-treated with RA, eliminated.

Task 10: Sequence cDNA inserts. Since the 2 genes of primary interest were actually identified using immunoblotting, it is not necessary to sequence the corresponding cDNAs. Therefore Task 10 is no longer relevant to the goals of this project.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that T47D cells can not be efficiently infected with retroviruses
- Identified PKC α and PTP-1C proteins up-regulated in retinoid treated cells
- Demonstrated that PKC α mediates the anti-proliferative action of retinoids in T47D cells
- Demonstrated that PKC α cooperates with retinoids to limit the proliferation of the normally retinoid resistant cell line, MDA-MB-231

REPORTABLE OUTCOMES

Cell lines constitutively expressing PKC α (derived from both T47D and MDA-MB-231 parental lines) and inducibly expressing PKC α (MDA-MB-231 only) as well as appropriate vector controls. In addition we have established an MDA-MB-231 cell line that expresses the lacI gene product that will be useful as a host for additional inducible expression experiments.

CONCLUSIONS

To date we have drawn two important conclusions. The first is a negative one. This is that the cell line initially chosen as a host for the enhancer trap cloning strategy is unsuitable for these experiments. This is the result of an as yet poorly understood resistance to expression of retroviral vector encoded genes. The second conclusion is more positive and enlightening. This is the finding that the alpha isoform of protein kinase C is retinoid induced in hormone dependent breast cancer cells and that the activity of this PKC is responsible for the retinoid-dependent disruption in mitogen signaling that results in growth arrest. The significance of this role of PKC α is underscored by our further finding that expression of PKC α in hormone-independent breast cancer cells allows retinoids to inhibit mitogenic signaling. In the long term,

these results might open the door for developing combination therapy approaches that jointly target retinoid and PKC signaling.

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